Executive Summary
New Energy Company of Indiana CRADA
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CRADA Background

The CRADA between the National Renewable Energy Laboratory and the New Energy Company of Indiana was initiated in April, 1991. The original Statement of Work called for a 3 phase effort over a 26 month period of time to investigate the use of technologies developed within the Biofuels Program at NREL for the conversion of lignocellulosic feedstocks to ethanol to New Energy's corn dry milling process for industrial ethanol production. The general objective was to improve the conversion efficiency of the existing dry milling process by developing process modifications to convert the fiber-based carbohydrates and/or unconverted starch to ethanol without sacrificing the value of co-products that are currently generated. More specifically, the technical probability and economic feasibility of various process modifications on a commercial scale were evaluated.

Phase 1 called for a preliminary process engineering evaluation of several process alternatives that were identified, based upon preliminary laboratory results using samples of distillers dried grains with solubles (DDGS) and stream flow and composition data from the existing New Energy process. The process alternatives identified in Phase 1 with the greatest economic potential, based upon assumed levels of conversion, were chosen to be evaluated in Phase 2 of the CRADA. Phase 2, a 10 month effort, involved a detailed laboratory-scale experimental evaluation of these process alternatives using New Energy process stream samples to determine actual process performance. Process economic evaluations were also updated, using the actual laboratory performance data. Following completion of Phase 2, New Energy designed, procured, and installed two 9000 L pilot fermentors. One of these was dedicated to ethanol yield improvements, represented by the work performed under the CRADA. Following completion of the fermentor installation, Phase 3 of the CRADA commenced. This work involved an number of pilot fermentations as well as shake-flask fermentations under identical conditions to verify the results obtained in the pilot fermentations. Again, process economic evaluations, based on the results obtained in the pilot fermentations, were performed and recommendations were made for any commercially viable process alternatives.

In September, 1994, a modification to the Statement of Work was negotiated. This resulted in a widening of feedstock options beyond just the fiber fraction of corn kernels to include other regionally-available lignocellulosic wastes--namely corn stover. In addition, process alternatives involving the conversion of corn fiber and residual starch were revisited in an approach that better integrates any process changes with the existing process to minimize any capital expenditures needed for process modifications. Although some work on corn stover was performed during this period, mainly in the development of dilute acid pretreatment methods, the majority of the work was focused on corn fiber and residual starch conversion options. A number of different approaches were investigated, including the use of blends of commercially available hydrolytic enzymes, such as cellulases and proteases, added in the current saccharification step to loosen up the structure of the fibrous carbohydrates, allowing for better accessibility of amylase enzymes in order to convert residual starch. In addition, the use of these hydrolytic enzymes was studied as an approach to lower in-process viscosity, aimed at improving current process. Finally, preliminary work involving hot water and dilute acid batch pretreatment methods on corn fiber that had been separated from current process streams.

Summary of Phases 1 and 2

Phases 1 and 2 under the original Statement of Work were conducted between May, 1991 and June, 1992. A large summary document entitled "New Energy Company of Indiana/NREL CRADA Phase 2 Final Report--June 1992" captures all experimental and process analysis results and conclusions in a comprehensive manner. Briefly, this document describes the 3 general process alternatives investigated in a conceptual manner in Phase 1. These configurations involve 3 different process streams as starting points for a process addition--the fermentation beer prior to distillation, the whole stillage bottoms exiting the distillation stripping columns, and the wet cake solids from the whole stillage centrifuges. Conceptual process configurations were devised for each of these alternatives and assumed performance yields and general process costing methodologies were used to project the economic potential of each alternative. Based upon these results, 2 process alternatives--utilizing the fermentation beer or the wet cake as feed streams, were recommended for laboratory scale evaluation in Phase 2.

Three main processing steps were evaluated for each process configuration in Phase 2--cellulase enzyme production, simultaneous saccharification and fermentation (SSF) of hexose carbohydrates, and a separate pentose fermentation to convert xylose and perhaps arabinose to ethanol. Prior to any laboratory work being initiated, extensive chemical analysis and methods development work was carried out on appropriate process samples to characterize the composition of the various process streams. In addition, carbohydrates were evaluated not only to determine their sugar make-up, but the source of those sugars (i.e. starch-based or lignocellulosic-based). This allowed for a determination of 100% of theoretical yields of ethanol from the various process streams, allowing for a benchmark to measure actual performance levels achieved during experiments.

A few general conclusions form each process area are as follows:

Simultaneous Saccharification and Fermentation

> Very little of the hemicellulose in either the beer stream or wet cake stream was hydrolyzed using the cellulase enzyme preparations (both commercial preparations and cellulase produced from New Energy process streams substrates). Thus, the ethanol potential is lessened by the unavailability of this fraction of carbohydrates (unless a pretreatment step is included). In addition there are significant levels of residual starch present, which can largely be converted if the amylase enzymes are "re-activated" in the fermentation beer stream by separating the solids (containing the insoluble lignocellulosic fiber and residual starch) and diluting to about 3 % (w/v) ethanol with recycled thin stillage. Additional amylase enzymes would have to be added to the wet cake option since the enzymes are inactivated by heat in the distillation process. Significant amounts of ethanol are available via the "dilute and hold" approach using the fermentation beer solids with no additional enzyme requirement. Incrementally higher levels of ethanol production is possible with the use of cellulase enzymes, but not enough revenue could be generated to offset the cost of either purchasing or producing the cellulase.

Pentose Fermentation

> Since the enzyme preparations tested did very little to hydrolyze pentose-containing carbohydrates, a pretreatment process would be necessary to release these sugars. Thus, much of the pentose

fermentation work was focused on the identification of pentose-fermenting microorganisms on ideal sugar substrates, their nutrient requirements in an environment of the New Energy process streams, ethanol tolerance, and tolerance to known inhibitory compounds in the New Energy process streams, such as lactic acid. It should be noted that all of this work was carried out on previously identified, naturally occurring pentose-fermenting microorganisms, i.e. this work pre-dated the development of engineered *Zymomonas mobilis* strains capable of co-fermenting glucose/xylose or glucose/arabinose mixtures. For reasons or co-product acceptance concerns, the engineered *E. coli* xylose- fermenting strains were not evaluated.

Cellulase Enzyme Production

Cellulase can be produced via various strains of *Trichoderma reesei* in significant quantities using New Energy fermentation beer or wet cake process streams. The use of the fermentation beer solids required near-complete removal of ethanol; otherwise, significant inhibition of both cell growth and enzyme production occurs. Acceptable enzyme productivities were not achieved using just New Energy process streams. The use of lactose as an inducer and a carbon source in a fed batch mode was required in conjunction with the New Energy process substrates. Adapted strains could grow well on hemicellulose and cellobiose-containing substrates, but did not produce significant quantities of enzymes capable of hydrolyzing those carbohydrates. Thus, it was concluded that the costs of enzyme production did not justify their use in this particular application--at least with the available *Trichoderma* strains tested.

Pretreatment

Although not part of the original Phase 2 experimental plan, it was recognized that enzyme preparations tested did not possess the capability to hydrolyze hemicellulose. Therefore, any attempt to convert pentose sugars to ethanol would require a pretreatment step to liberate these pentose sugars. Preliminary pretreatment evaluations using dilute sulfuric acid in an autoclave were performed and a function describing the impact of time, temperature, and acid concentration on pentose sugar yield was developed. This function, extrapolated to higher temperatures, suggested that near theoretical yields of pentose sugars could be obtained using a dilute sulfuric acid pretreatment approach with these substrates. This work served as the basis for corn fiber pretreatment work conducted during later phases of the CRADA.

Process Economics and Overall Conclusions

After all of the experimental data was considered, the process economic evaluations suggested that an approach where the fermentation beer solids were separated, diluted with thin stillage to an ethanol concentration of 3% (w/v), and held under fermentation conditions for 48 hr, would result in a significant increase in ethanol yield from residual starch (on the order of 6 million gallons of additional ethanol produced per year). This was the approach recommended for pilot-scale evaluation in Phase 3 of the CRADA.

New Energy Pilot Plant Experiments and Evaluation

After Phase 2 was completed in July 1992, a joint decision by New Energy and NREL was made to proceed forward with Phase 3, calling for the design, construction and installation of a pilot facility at New Energy to

test leading process options with sidestreams taken directly from the current process at the pilot scale. After a 12 month design and construction period, the pilot fermentor dedicated to ethanol yield improvement work was commissioned and actual runs were conducted beginning in September, 1993.

Three pilot scale fermentations and associated shake flask duplicated were conducted as part of this campaign. Although the recommendation from Phase 2 was to use fermentation beer solids without cellulase addition, it was decided to use whole stillage in these pilot fermentations. An effective means of separating the solids out of the fermentation beer was not available at the pilot scale and the ability to adequately sterilize the pilot fermentation system was limited. The whole stillage, coming directly from the distillation columns bottoms, was reasonably sterile and the operational difficulties of transferring this material from the main process flow to the pilot fermentor was minimal.

Pilot Fermentation 1

Fresh whole stillage along with the Genencor Spezyme CP cellulase enzyme (10 FPU/g cellulose) and fresh yeast inoculum were used for the pilot fermentation. The sterilized fermentor was charged with 3800 L of whole stillage, pH adjusted to 5.0, and cooled to 50°C. After cellulase addition, the fermentor was cooled to 38°C (about 1.25 hr) and then inoculated with New Energy fresh liquid yeast concentrate. The fermentor was run for approximately 120 hr. Due to inadequate mixing in the pilot fermentor, which could result in a slow distribution of enzyme and yeast throughout the entire broth volume, slow initial rates of ethanol production were observed. However, the ultimate levels of ethanol produced are virtually identical in the pilot fermentor and duplicate shake flasks, indicating that results achieved in the pilot fermentor are quite scalable from the shake flask level. An additional shake flask study investigating a number of different fermentation conditions and enzyme loadings revealed that the addition of glucoamylase enzyme along with the cellulase results in much higher levels of ethanol production. Presumably, residual starch or starch-based oligomers are being converted along with cellulose or cellulose-based oligomers. In addition, the conversion of the starch fraction may make the remaining cellulose more accessible to the cellulase enzymes, further increasing the production of ethanol from cellulose.

Pilot Fermentation 2

The prime change in the second pilot fermentation run involved the use of glucoamylase along with cellulase. Operationally, the pilot fermentation was run identically to Pilot Fermentation 1, except that modified piping in the fermentation vessel to facilitate mixing installed and pH in the fermentation vessel was controlled at 4.8 throughout the run. Significantly higher levels of ethanol were produced, both in the pilot fermentor and in shake flask as those obtained in the cellulase alone flask. The enhanced conversion of residual starch is believed to be responsible for this improvement. The pilot fermentation did not perform as well as the shake flask duplicate. The reason for this is unknown, but contamination is a possibility. The associated shake flask study indicated that glucoamylase alone performed better than cellulase alone, but not as well as cellulase/glucoamylase blends. The use of protease, either as a separate treatment prior to addition of other enzymes or as a constituent in the enzyme blend, did not appear to produce any beneficial results.

Pilot Fermentation 3

The major change is this run involved using the Hydrasieve Static Screen to generate higher solids material, which would result in higher ethanol concentrations at a given yield, reducing fermentation

volumes and distillation costs. Again, cellulase and glucoamylase were employed in the pilot fermentor, based upon the positive results seen in Pilot Fermentation 2. In this run, pilot fermentor yields were significantly lower that identical shake flask runs. It was felt that the solids level employed in the pilot fermentation were too high for the pump-around mixing loop, resulting in poor mass transfer and poor temperature control. A shake flask study indicated that lower glucoamylase loadings, in conjunction with cellulase, resulted in equal (if not higher) yields than a full dosage of glucoamylase.

Economic Analysis and Conclusions

The clear economic drivers in determining whether a commercially viable process addition exists are the required loading and cost of added enzymes traded off against the additional revenues realized from higher ethanol yields. Capital equipment costs and other additional operated costs also need to be included in the economic evaluations. As producing cellulase enzymes on-site was not regarded as a practical possibility, the costs of cellulase are strictly related to the current market value of commercial cellulases. These costs are generally in excess of $5.00/L (each L contains ~85,000 FPU). As noted in the Section 2 reports, at baseline cellulase and glucoamylase loadings, cellulase costs need to be about $0.85/L and overall conversion of cellulose and residual starch to ethanol needs to be 90%. These yield levels were achieved in shake flasks, but not in the pilot fermentor at the higher assumed solids loading due to mixing and temperature control difficulties. However, the required cellulase costs are clearly not reasonable in the current cellulase marketplace. These results led to a basic change in philosophy in the CRADA, which resulted in the approach stated in Modification 2, where the emphasis was place on using just small amounts of cellulase and/or protease in conjunction with current amylase usage to more cost-effectively target residual starch conversion. In addition, minimizing capital expenditures on any process changes was emphasized, resulting in process options that better utilized existing process unit operations.

Waste Lignocellulosic Feedstock Availability in the South Bend Area

In 1994, with the original Statement of Work set to expire, New Energy and NREL negotiated a modification of the CRADA. One of the new area of interest was a broadening of feedstock options beyond the corn fiber that had been investigated in early phases of the CRADA. A study was commenced to evaluate the possible waste lignocellulosic feedstock options available in the South Bend area.

The definition of waste lignocellulosic feedstock used in this report is the same as defined in the New Energy/NREL CRADA Modification 2. Any feedstock that is paper or wastepaper derived was not considered for either this report or the CRADA in general. Thus, waste lignocellulosic feedstocks include agricultural residues, municipal and industrial residues that are not paper or wastepaper derived, lumber/sawmill wastes, food wastes, and yard/garden wastes.

This study recommended that two categories of feedstocks were available in large quantities and could be evaluated in future laboratory experiments. These categories include corn related agricultural residues, such as corn cobs and corn stover, and yard and garden wastes, such as those currently deposited on New Energy property by the City of South Bend. Due to resource constraints and the ties to the existing feedstock in the New Energy process, it was decided that any experimental evaluation would focus on corn stover and not the yard/garden wastes.

Economic evaluations regarding the collection, transport, and storage of corn cobs and corn stover indicate that farmers in the Indiana-Michigan region will have to receive $50-65/dry ton in order for them to harvest large quantities of such materials. This cost may seem to be too high to support any economically viable operation for ethanol production from these materials, but a number of changes in areas such as harvesting techniques and less intensive tillage practices may ultimately result in a significantly lower cost of feedstock procurement. The ultimate goal of full utilization of the entire unfractionated corn plant may result in quite simple, inexpensive harvesting methods.

Pretreatment of Corn Stover

In conjunction with the recommendation that corn stover be evaluated as a possible additional feedstock, a preliminary study to identify pretreatment conditions to liberate sugars from this feedstock was conducted.

A series of dilute sulfuric acid pretreatment experiments was conducted in a Parr reactor using 2 mm screened corn stover. The best conditions identified via a screening experiment were 170 ^{o}C, 10 minute reaction time, and 0.8% (w/v) sulfuric acid. Over 78% of the original xylan was hydrolyzed to xylose, and about 12% of the original cellulose was hydrolyzed to glucose. These results are fairly typical for this type of biomass feedstock. Formation of sugar degradation products was relatively minor.

Simultaneous saccharification and fermentation (SSF) experiments were conducted to evaluate the enzymatic digestibility and ethanol potential of cellulosic solids resulting from the pretreatment reaction. Both standard NREL SSF yeast strains and the New Energy production strain were evaluated, and two different cellulase enzyme loadings (10 and 25 FPU/g cellulose) were tested in a media with a cellulose content of 3% (w/v). Both yeast strains performed similarly at the higher cellulase loading, achieving about 85% ethanol yields in 72 hr and >95% ethanol yields in 168 hr. At the lower enzyme loading, the New Energy yeast outperformed the NREL SSF yeast. Although initial rates of ethanol production were slower, the New Energy yeast system was able to match the 95% ethanol yields seen at the higher enzyme loading in 168 hr. The NREL SSF yeast system was only able to achieve about 80% ethanol yields in that time frame. The reason for the superior performance of the New Energy yeast at low cellulase loadings is not clear.

After the completion of this preliminary study, the focus of CRADA work shifted back to corn fiber conversion, so the follow-on activities for corn stover recommended at that time were not conducted. These recommendations included the evaluation of pretreated corn stover with solubilized xylose and arabinose in the liquid fraction in a combined simultaneous saccharification and co-fermentation (SSCF) using engineered *Zymomonas mobilis* strains with pentose fermentation capability. In addition, the pretreatment of corn stover in a flow-through, countercurrent mode should be explored. Countercurrent pretreatment schemes on other feedstocks have yielded highly digestible solids, although the issue of prehydrolyzate toxicity and fermentability has not yet been fully addressed. Process configurations that utilize experimental data resulting from a countercurrent pretreatment should be compared to those from a batch (Parr reactor) pretreatment and be evaluated from an overall process engineering viewpoint. This could be done in conjunction with a countercurrent pretreatment of corn fiber (discussed in a later section), where the protein contained in the corn fiber could be extracted and recovered as a high-value co-product prior to dilute acid pretreatment for carbohydrate hydrolysis. Recent advances in the area of dilute acid countercurrent pretreatment shows promise in hydrolyzing not only hemicellulose, but at least a significant amount of the cellulose as well. This could reduce or even eliminate the need for cellulase enzymes in these types of processes, which has been shown to be a major cost obstacle in commercializing these technologies.

Use of Hydrolytic Enzymes for Residual Starch Conversion

One of the major changes in the direction of the CRADA was to refocus the conversion of corn fiber on approaches that minimized the requirements of cellulase enzymes and to focus on processes that better integrate with the existing New Energy liquefaction, saccharification, and fermentation unit operations. In Section 5, a series of presentation graphics is shown where an enzymatically enhanced starch conversion process is discussed. In this approach, relatively small quantities of hydrolytic enzymes (cellulase and/or protease) are added to either the mix tank prior to liquefaction or to the mix tank prior to saccharification. In order to maximize the residence time of the enzymes prior to fermentation, it would be preferable to add the enzymes to the liquefaction part of the process. However, the high liquefaction temperatures would inactivate protease and cellulases, which are optimal at temperatures of 40-60 ^{o}C. A study was undertaken to evaluate the addition of various blends of commercially available cellulases and proteases to samples of saccharification tank slurry. These samples were shipped frozen to NREL and were thawed just before each experiment. The appropriate amount of glucoamylase was added to simulate the current process and then varying amounts of cellulase and protease were added. The slurries were allowed to incubate at the temperature and residence time to simulate the saccharification tank. After conducting initial screening to determine the best available commercial cellulases and proteases for this particular application, the general approach taken was to determine if relatively small quantities of cellulase and/or protease would be a cost effective approach to increasing ethanol yields by loosening up the structure of the corn fiber, allowing for the accessibility and conversion of bound residual starch by alpha-amylase and glucoamylase that have previously been added to the process. Experimental results and economic analysis indicated that although some small ethanol yield increases can be measured under certain conditions, the costs of the enzymes at the levels needed to observe a yield increase are too high to be cost effectiveAs found with previous work in the CRADA, costs of commercial cellulases are too high for cost-effective use in this manner.

As previously mentioned, the current liquefaction process is too hot for utilization of mesophilic cellulases from *Trichoderma reesei*. Even the saccharification process is above the optimum temperature of ~55 ^{o}C for these enzymes. However, one of the advanced technology area being developed within the DOE/NREL Biofuels Program is the evaluation of thermophilic cellulase enzyme systems, capable at operating at temperatures significantly higher that cellulases from *Trichoderma reesei*. Along with many other applications, thermophilic cellulases may have application in the liquefaction and/or saccharification steps in a dry milling process.

In particular, a thermal stable recombinant engineered endoglucanase, E1, from *Acidothermus cellulolyticus*, which is active at temperatures of up to 80 ^{o}C, was used in studies where small amounts were added to simulated liquefaction and saccharification process using appropriate New Energy process samples. It was found that when E1 is added to samples which are at temperatures of 60 to 70 ^{o}C and this is followed by SSF, an increase in the glucose utilization rate in the E1-containing samples is readily observed. This appears to be dose-dependent since decreasing the E1 content by half, decreases the rate accordingly. Temperatures of 90 ^{0}C appear to decrease E1 activity , but E1 appears to continue to be active at SSF temperatures. Since the viscosity of these slurries is decreased by cellulase addition (*T. reesei* and E1), it could be conceived that diffusion of available glucose could be enhanced in these samples, therefore increasing glucose uptake by yeast. It is also possible that the E1 is fairly active at these lower SSF temperatures and is actively depolymerizing exposed cellulose in the starch matrix, therefore causing increased hydrolysis of starch to glucose during the first 24 hours of SSF. Theoretically, this is supported by the observation that E1 does appear to cause a decrease in the glucan content of all E1-containing samples. Additionally, analysis of final SSF supernatants indicates that the E1-containing samples have slightly more soluble polymeric glucans than

the control samples.

Use of Hydrolytic Enzymes for In-Process Viscosity Reduction

In early 1996 a study investigating to potential use of hydrolytic enzymes to reduce in-stream process viscosity between the saccharification and fermentation units was initiated. Most experiments were conducted using saccharification tank samples with glucoamylase and small amounts of cellulase and/or protease added. The samples were incubated at ~60 ^{o}C for 2 hr to simulate saccharification tank conditions and viscosity was measured and compared to a control (no cellulase or protease added). Viscosity was measured with a Stormer viscometer, which utilizes a paddle spindle to keep solids suspended. The results of these experiments suggest that the amount of viscosity reduction is dependent on several variables including solids content, supernatant volume, temperature, incubation time, shear (flow) rate, composition and enzyme activity. When compared to the control samples, the viscosity reduction observed for glucoamylase, cellulase, and protease enzyme levels is not as great at lower dosages. This was observed in the protease/cellulase samples and with the protease only samples.

The viscosity reduction attained with "protease only" is minimal. It is possible that the protease dosage caused a decrease in the amount of glucoamylase and inactivated amylase in the sample, which would account for the increase in drop time. A larger decrease in viscosity was attained in the "cellulase only" experiments, than in the experiments conducted with "both" cellulase and protease and with "protease only". This result would indicate that use of protease should be omitted when looking for reductions in viscosity. The greatest reduction was observed at a moderate enzyme loading (~5 FPU/g cellulose). A significant reduction was also observed with a 1 FPU/g cellulose dose.

Earlier, it was noted that thermophilic E1 endoglucanase was tested to determine if that enzyme component could improve the saccharification process. With the initiation of the viscosity reduction study and the positive impact of mesophilic cellulases on measured process stream viscosity, some preliminary work was conducted in a similar manner with the E1 endoglucanase. Using a capillary viscometry method to minimize sample volume and recombinant enzyme usage (rather than the Stormer viscometer method, results indicate that the addition of E1 to corn slurries decreases the viscosity of the liquid fraction of this suspension. This decrease in the liquid fraction viscosity may explain the increase in glucose uptake rates (and higher ethanol yields) previously noted in SSF experiments with the addition of the E1 endoglucanase.

Formation of Transglycosylation and Sugar Reversion Products in the New Energy Process

An aspect of fully integrated plant operation not always considered is the consequence of allowing sugars, especially mono- and disaccharides, extended residence times following initial prehydrolysis or enzyme saccharification. Reducing sugars are reactive under virtually all conditions encountered in a corn milling or biomass conversion facility. Mildly acidic conditions and moderate temperatures permit reversion and transglycosylation of glucose to generate populations of all possible configurations of alpha- and beta-linked disaccharides (i.e., 1-1, 1-2, 1-3, 1-4, and 1-6) and even higher-order oligomers in yields that approach 10%. Many of these oligosaccharides are not fermentable, and the nonreducing sugars, such as 1-1 beta- and alpha-linked glucose (a- and b-trehalose), are quite stable even after dilution and neutralization.

When enzymes are added to the process, reversion and transglycosylation reactions produce all possible configurations of disaccharides consistent with the anomeric requirement of the enzyme; i.e., a-glucosidase

produces only alpha products and b-glucanases produce only beta products, but many linkage combinations are possible. Clear precedent exists for efficient production (20% to 37% yield) of transglycosylation products from 10% solutions of glycosyl donor (cellobiose) using a *Fusarium oxysporum* b-glucosidase and from 10% solutions of maltose using an *A. niger* a-glucosidase. Enzymatic transglycosylation is also possible at glycosyl donor concentrations as low at 2%. Because most amylases and cellulases have active sites that can tolerate some diversity in the glycosyl acceptor group, it may be possible to find disaccharides or higher forms of glucose-xylose, glucose-galactose, and even glucose-mannose in biomass hydrolysis process streams. Of further concern to bioethanol plant efficiency are the observations that transglycosylation reactions that involve glycosyl transferases have been reported that utilize non-carbohydrate species as glycosyl acceptors, including alcohols (methanol, ethanol, and propanol) and lignin model compounds (veratryl and vanillyl alcohols).

The dry milling of corn includes a saccharification process that is preceded by liquefaction of the corn at extremely high temperatures. The incubation time of saccharification varies, but it is normally between 4 - 8 hours. Transglycosylation reactions occur most often during the saccharification process, when glucose levels can reach 90-95% syrups after total saccharification. To avoid the formation of reversion products, saccharified streams are fed into fermentation tanks after 4 - 8 hours of exposure to glucoamylase. Our experiments indicate that condensation products begin to be formed before 4 hours, in experiments using clean sugar streams (data not shown). Although most commercial glucoamylase preparations today do not contain transglucosidase activity, glucoamylase itself has been shown to form condensation products such as a-trehalose and nigerose.

We have performed preliminary evaluations of New Energy process streams to detect the presence of disaccharides formed during saccharification. These preliminary results indicate the presence of disaccharides in the existing streams which have not or are not fermented by yeast. The preventable loss of fermentable sugars to the formation of condensation products such as these should be the focus of future research. We have identified specific types of transglycosylation and reversion products located in New Energy process streams. It is clear that a better understanding of conditions that lead to the formation of these products, and a modification of the process to minimize their formation, can lead to higher fermentable sugar and ethanol yields.

Pretreatment of Corn Fiber

Evaluation of various cellulase preparations (both commercial preparations and cellulases produced from *Trichoderma reesei* fermentations using New Energy process streams as carbon sources) revealed that all tested cellulase sources do not possess sufficient hydrolytic action on hemicellulose to effectively release sugars from this fraction of corn fiber. Therefore, in order to utilize hemicellulose sugars from corn fiber, a thermochemical pretreatment process is necessary.

Preliminary studies were conducted in which a batch dilute sulfuric acid pretreatment process on selected New Energy corn-fiber containing streams was evaluated. These experiments were conducted using centrifuged solids from saccharification tank samples prior to glucoamylase addition. Experiments were conducted in a 1 L Parr reactor at a 10% (w/v) solids concentration. Initial studies focused on a hot water approach (no added sulfuric acid), as the lignocellulosic fiber had already been hydrated during the liquefaction step. Although pretreatment temperatures in the range of 170-210 ^{o}C and residence times from 5-30 minutes were tested, acceptable yields of sugars from the hemicellulose fraction were not obtained. Conditions severe enough to result in near-complete hydrolysis of hemicellulose caused excess formation of

sugar degradation products, such as furfural. Less severe conditions resulted in only a limited hydrolysis of hemicellulose. The hot water approach was able to hydrolyze about 85-90% of the starch, but most of the resulting carbohydrates were in the form of oligomeric glucans rather than monomeric glucose.

A series of dilute sulfuric acid batch pretreatments was also conducted. The residence time in these experiments was fixed at either 5 or 10 min. The temperature range was 160-180 ^{o}C and the sulfuric acid concentration ranged from 0.1-0.5% (w/w). Significantly higher levels of hemicellulose hydrolysis was achieved, and the proportion of oligomers, both from residual starch and hemicellulose, was reduced significantly. However, limitations in the mixing capabilities of the bench-scale pretreatment apparatus allowed a solids level in the pretreatment vessel of only 10% (w/v). This results in relatively large pretreatment reactor requirements, high heat demands, and dilute sugar streams resulting from the pretreatment reaction. Further work should focus of pilot-scale reactor configurations capable of processing much higher solids loadings.

One important consideration in the pretreatment of corn fiber is the fate of the protein, which is a major component in corn fiber streams and ultimately provides significant co-product revenues in the form of DDGS. Prior to this work, the impact of high temperature, acidic environments on the recoverability and value of the protein was largely unknown. Preliminary work conducted in this study seems to indicate that protein loss in the pretreated solids is not a major consideration. However, nothing is known at this point about the relative amino acid levels in the pretreated solids as compared to current DDGS, or the nutritional value or palatability of such a co-product. Detailed peptide and amino acid analysis on pretreated corn fiber should be conducted, ultimately leading to a feeding trial study.
Finally, a new approach in biomass pretreatment technology should be evaluated on corn fiber substrates. This approaches uses a flow-through, countercurrent contacting of solids and liquids in two or more stages to achieve a higher yield of hemicellulosic sugars from hemicellulose and a more digestible cellulosic solid. In addition, recent advances indicate the possibility of hydrolyzing a significant fraction, if not all, of the cellulose component to recoverable glucose, reducing or even eliminating the need for an enzymatic hydrolysis step. For a relatively easy-to-hydrolyze feedstock like corn fiber, such a process could be a real possibility. Also, by using known protein extractants such as ethanol with sodium hydroxide, an extraction of protein prior to the pretreatment of the remaining lignocellulosic fiber becomes a practical possibility. The flow-through reactor configuration would allow for both a protein extraction and a fiber pretreatment in the same equipment simply by feeding different liquids through the solid fiber particles. Not only would this approach eliminate any protein degradation that might occur under pretreatment conditions, but it could provide an opportunity to capture the protein in a clean, concentrated form that may be more valuable that the current DDGS co-product.

Overall Conclusions and Recommendations

A large number of process scenarios were investigated and evaluated as part of the technology development work conducted under the New Energy CRADA. Significant knowledge on the issues related to converting lignocellulosic corn fiber, residual corn starch, and corn stover was gained. Many process options investigated were found to not be cost-effective options at the present time, based upon evaluation of additional revenue streams that could be generated as compared to the costs associated with the process additions. However, those technologies that are not cost effective today should be re-evaluated on a regular basis in the future, as both technology improvements and economic factors could dramatically improve the viability of some of these options.

However a few process alternatives studied appear promising enough today to warrant serious consideration, either for plant trials or for more detailed study to determine commercialization potential. The leading candidate for a plant trial involves the utilization of small quantities of commercial cellulase for viscosity reduction. It has been demonstrated that a measurable reduction in viscosity is achievable at low cellulase loadings. Although reduced viscosity is expected to have a positive impact on process performance, the only way to accurately quantify the impact is to conduct a plant trial. This would provide the necessary information to determine whether this use of cellulase enzymes is cost-effective.

Another area of promise that requires further development is the pretreatment of corn fiber, especially in conjunction with the extraction of higher value protein concentrates. The batch corn fiber pretreatment work conducted under the CRADA showed promise in liberating sugars from virtually all of the residual starch, as well as a significant fraction of hemicellulosic sugars (xylose and arabinose) using dilute sulfuric acid. Other dilute acid pretreatment approaches, specifically, the countercurrent flow-through process, can further improve the hemicellulosic sugar yields and produce a highly digestible cellulosic solids for enzymatic conversion to glucose. Recent advances have indicated the possibility of also hydrolyzing the cellulose as well as the hemicellulose and residual starch, eliminating the need for costly cellulase preparations. In addition, the flow-through approach allows for the possibility of extracting out a significant fraction of protein in a clean and concentrated form prior to the pretreatment process--using the same reactor for both protein extraction and pretreatment. This type of process could have major impacts on revenue streams and profitability. Ultimately, corn stover could also be included in this pretreatment approach, thus broadening feedstock choices when corn prices become high.

Although a longer term technology, the continued development of thermophilic cellulase components should continue to be monitored and as more efficient production systems are developed, their performance and economic impacts on the New Energy process should be evaluated. Thermophilic cellulases are a natural match for the high temperature liquefaction and saccharification process already in place. A process where sugars in high concentration from both corn starch and corn fiber, without yield losses from transglycosylation and reversion product formation, could be possible with a thermophilic cellulase and amylase system in a properly controlled and configured process.